7

Staining Principle and General Procedure of Staining of the Tissue

7.1 Introduction

The tissue section is colourless because the fixed protein has the same refractive index as that of glass. We use dyes that have specific affinity with the different tissue proteins and colour them differently. This helps us to understand the morphology of tissue.

7.1.1 Dyes Used for Staining

The dye may be natural or synthetic. The natural dye is extracted from plants and animals. Nowadays natural dye is rarely used except haematoxylin and carmine. The majority of the synthetic dye is petroleum derivatives. All these synthetic dyes have a central benzene ring (Fig. 7.1). The benzene has chemical formula C_6H_6, and it is in a ring form which is very flexible. Benzene is colourless; however if certain chemical group is inserted into the benzene ring, then it will impart colour, such as when two H atoms in benzene ring are replaced by two O atoms, then the compound quinone ($C_6H_4O_2$) is formed which is a chromogen. This grouping in the benzene ring that imparts colour is known as chromophore, and the chemical compound formed by the grouping is known as chromogen.

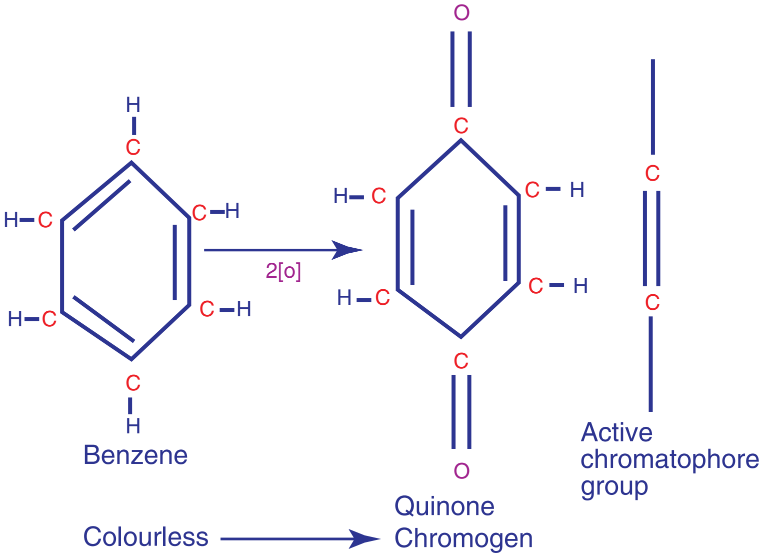

Fig. 7.1 Benzene ring. Benzene itself is colourless. When two H atoms in benzene ring were replaced by two O atoms, then the compound quinone ($C_6H_4O_2$) is formed which is a chromogen

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How Dye Produces Colour The visible light has the range of wavelengths between 400 and 650 nm. The white light contains all the seven colours (VIBGYOR: violet, indigo, blue, green, yellow, orange and red) with the wavelength in between 400 and 650 nm. A chromogenic dye absorbs the light of particular wavelength of the white light representing a specific colour and emits the light containing the rest of the colour. Therefore we see the particular colour of the dye,such as the dye that absorbs red light will be visible as green coloured in the naked eye.

The dye is the most elementary component of staining of a tissue. In general dye has two components: chromophore and auxochrome part (Fig. 7.2):

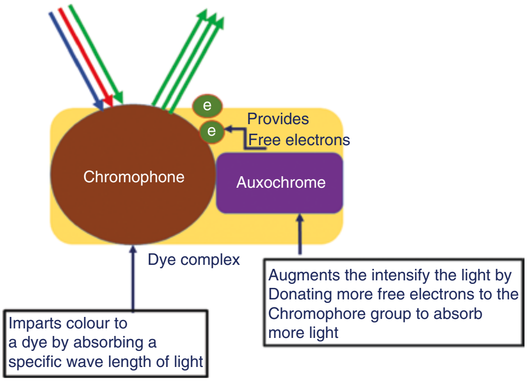
e
e
Provides
Free electrons
Chromophone
Auxochrome
Dye complex
Augments the intensify the light by Donating more free electrons to the Chromophore group to absorb more light
Imparts colour to a dye by absorbing a specific wave length of light

Fig. 7.2 Schematic diagram showing colour generation by dye. The chromophore group absorbs light and imparts colour to the stain. The auxochrome group donates more electrons to the chromophore group and helps to absorb light of longer wavelength in the visible range

- *Chromophore groups*: The chromophore group absorbs light and imparts colour to the stain. These groups have many free electrons that absorb the ultraviolet rays of light which are not in the visible range.
- *Auxochrome part*: This part of the dye helps to intensify the light. It is an ionizing group that also helps to stick the stain with the tissue. The auxochrome group augments more free electrons in the chromophore groups. The increased number of electrons in the system helps to absorb light of longer wavelength in the visible range.

7.1.2 Types of Dye

The dye can be classified on the basis of electrical charge (Table 7.1):

(a) Anionic dye or acid dye
(b) Cationic dye or basic dye
(c) Neutral dye
(d) Ligand dye (chelating)

(a) *Anionic dye or acid dye*: These dyes carry negative charge (coloured anions), and in an electrical field, they migrate towards anode. The dye is mostly of low molecular weight and soluble in water. Most of the dyes have two or more anionic groups which makes them soluble in water. These acid dyes combine with the tissues that carry positive charges, and those tissues are called as “acidophilic”.

Example: Eosin is one of the most commonly used anionic (acid) dyes.

Table 7.1 Types of dye based on electrical charge

Types of dye	Charge of the dye	Tissue to bind	Example
Anionic dye or acid dye	Negatively charged	• Cytoplasmic proteins • Collagen	Eosin
Cationic dye or basic dye	Positively charged	• Nucleic acid • Epithelial mucin	Methyl green, ethyl green and Alcian blue
Neutral dye	Contain both acid and basic dye	• Both nucleus and cytoplasm	Giemsa
Ligand or chelating dye	Weak acid so anionic and negatively charged	• Nucleus and cytoplasm	• Al ligand with haematoxylin: Harris haematoxylin and Mayer’s haematoxylin • Fe ligand with haematein: iron haematoxylin

(b) *Cationic dye or basic dye*: Cationic dye carries a positive charge (coloured cation) and move towards cathode in an electrical field. The cationic dyes are mostly soluble in ethanol. These are basic dyes and combine with tissues that carry negative charge. This negatively charged tissue combining with basic dye is called as "basophilic" tissue.

Example: The examples of basic dye are methyl green, ethyl green and Alcian blue.

(c) *Neutral dye*: These are the compound dyes that contain both acid and basic dyes in combination. In aqueous solution, the acid dye and basic dye exchange electrons and combine together to precipitate in the tissue. Romanowsky-Giemsa staining is the best example of the compound dye that undergoes electron transfer process. Romanowsky-Giemsa contains azure B and eosin Y. The azure B dye is the electron acceptor, and eosin Y is electron donor.

Example: Giemsa

(d) *Ligand or chelating dye*: Ligand dye is the complex compound that consists of dye and a metal ion. They are also known as metallochrome. They are usually weak acids. Haematein is the oxidized haematoxylin, and it is used as a combination of aluminium (Al) ions or iron (Fe) ions. The metal-ion complex has surplus charge that increases the solubility in water and makes the dye insoluble in alcohol, and therefore dehydration due to ethanol does not occur during staining.

Al^{3+}-Haematein It is a type of ligand dye where the metal aluminium (Al) is combined with haematein. It is used in Harris haematoxylin and Mayer's haematoxylin. As Al^{3+}-haematein is insoluble in ethanol, it can be used along with other anionic dyes, and both dyes are retained even after dehydration with alcohol.

Iron Haematein (Fe^{2+}-Haematein) Iron haematin is the ligand dye that consists of Fe and haematein. The dye is made by combining iron salt and haematoxylin. Fe binds with haematoxylin molecule by oxidizing it to haematein. Iron haematein is used for staining myelin and elastic fibres.

7.1.3 Types of Dye Based on Chemical Structures and Chromophore Groups

1. *Azo dye*: These dyes contain –N═N– chromophore group. Majority of the azo dyes are anionic (acid) dye. Example: orange G and Congo red
2. *Thiazine dye*: Thiazine dye contains –C–N═C– and –C–S═C chromophore group. Example: toluidine blue and methylene blue
3. *Triphenylmethanes*: Triphenylmethanes contain ═N– chromophore group. Example: methyl violet, light green and malachite green
4. *Azin dye*: This group of dye contains C–O═C and C–N═C chromophore. Example: Celestine blue and Nile blue sulphate
5. *Diphenylmethanes*: They contain –NH chromophore group. Example: auramine
6. *Xanthene dyes*: Example—eosin, Rose Bengal and phloxines
7. *Oxazine dyes*: They contain C–O═C chromophore group. Example: cresyl violet and Celestine blue
8. *Acridine dyes*: The dyes of this group are derived from acridine. Example: acridine orange
9. *Anthraquinone dyes*: These dyes are derived from anthraquinone. Example: carminic acid

7.2 Mechanisms and Theory of Staining

The staining is the combination of a coloured substance (dye) with the tissue that retains the dye after washing. The staining is primarily a chemical reaction between the dye and the tissue. The following chemical reactions are involved between the dye and tissue components (Box 7.1) [1, 2]:

1. Electrostatic bond
2. van der Waals attractions
3. Hydrogen bond
4. Covalent bond
5. Hydrophobic bond
6. Dye aggregations

Box 7.1 Mechanism of Staining

- *Electrostatic bond*
  - Coulombic force
  - The electrostatic bond between two oppositely charged particles: dye and tissue
- *van der Waals attractions: weak non-coulombic force*
  - Dipole-dipole interaction
  - Dipole-induced dipole interaction
  - Dispersion or London force
- *Hydrogen bond*
  - Weak bond
  - Type of covalent bond
- *Covalent bond*
  - The two electrically neutral atoms share electron with each other.
- *Hydrophobic bond*
  - Misnomer as in standard chemistry, there is no such bonding
  - Probably a type of van der Waals force
- *Dye aggregations*
  - Dye molecules aggregate in solution and then penetrate into tissue.

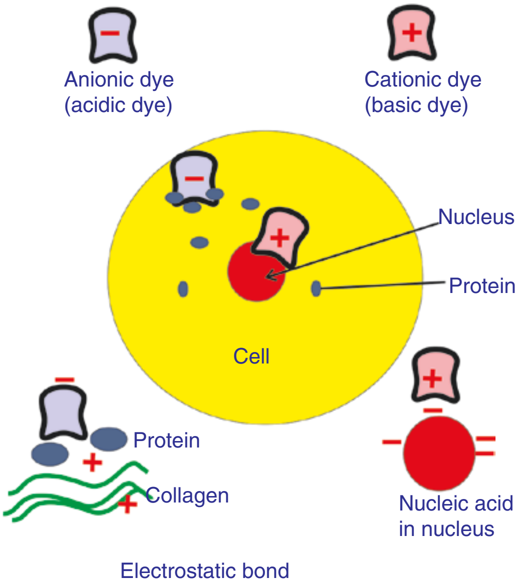

Fig. 7.3 Schematic diagram showing interaction of anionic and cationic dye with the oppositely charged tissue components. Acid or anionic dye binds with the cytoplasmic protein and collagen, whereas the basic or cationic dye binds with nucleic acid of the nucleus

1. *Electrostatic bond*: The electrostatic bond occurs between two oppositely charged particles, and coulombic forces work between the particles. The oppositely charged dye binds with the tissue (Fig. 7.3). For example, acid dye containing the anionic (negative charged) chromogen binds with the acidophilic tissue containing positive charge. Similarly the basic dye having a positively charged cationic chromogen binds with the basophilic tissue containing negative charge.

   The dye is the combination of chromogen and auxochrome that are oppositely charged. In the solution, the dye is dissociated into oppositely charged chromogen and auxochrome, such as basic dye which dissociates into cationic chromogen (positive) and anionic auxochrome (negative). Now the cationic positively charged chromogen of the basic dye combines with negatively charged tissue

   Example: Eosin, the acid dye, stains the cytoplasmic proteins.
2. *van der Waals attractions* (Fig. 7.4): This is a type of non-coulombic force. This is the weakest force due to the intermolecular interactions. The strength of this force is only 0.4–4 kJ/mol compared to 20 kJ/mol in ionic bond. When the electrons of an atom concentrate in one pole of the atom, then a dipole is formed. This dipole is just like a magnet having two poles.

   Dipole-dipole interaction: The positive charge of a permanent dipole interacts with other permanent dipoles, and electrostatic interaction occurs.

   Dipole-induced dipole interaction: Similarly a permanent dipole may induce adjacent atom and induces dipole. This permanent dipole may interact with induced dipole.

   Dispersion or London force: Permanent dipole may induce the adjacent atom as

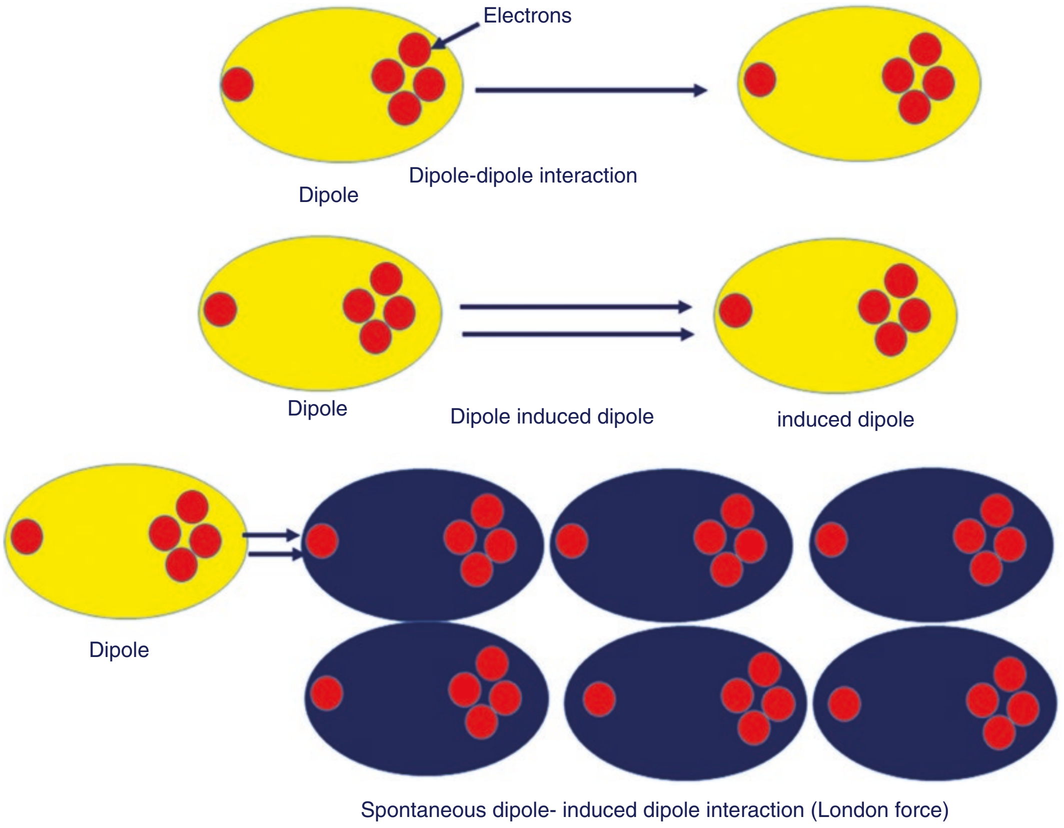
Electrons
Dipole
Dipole-dipole interaction
Dipole
Dipole induced dipole
induced dipole
Dipole
Spontaneous dipole- induced dipole interaction (London force)

Fig. 7.4 Schematic diagram of van der Waals force. The positive charge of a permanent dipole interacts with other permanent dipoles. In case of dipole-induced dipole interaction, the permanent dipole interacts with induced dipole. In case of London force, the permanent dipole induces the adjacent atom as induced dipole that further induces a chain of induced dipole and forms a large network of tissue with induced dipole interaction

induced dipole. The induced dipoles further induce a chain of induced dipole. In this way a large network of tissue may undergo induced dipole interaction. This is known as dispersion or London force.

Example: elastin stain by Miller's stain and Congo red stain.

3. *Hydrogen bond*: Hydrogen bonding is a weak bond. It is a type of covalent bond that occurs between hydrogen and a strong electronegative atom commonly O, N or F. Water forms hydrogen bond and so competes with stain-tissue bonds. Therefore, hydrogen bonding in dye-tissue less likely occurs in aqueous solution. Example: Best's carmine dye to stain glycogen.
4. *Covalent bond*: In case of covalent bond, the two electrically neutral atoms share electron with each other to satisfy the outer shell's required number. The covalent bond is a stronger bond.

   Example: periodic acid Schiff's staining for glycogen and Feulgen reaction.
5. *Hydrophobic bond*: This is a misnomer as in standard chemistry, there is no such bonding. It is probably a type of van der Waals force. When two hydrophobic molecules interact, then London force, the dispersion type of van der Waals force, interacts. Therefore instead of hydrophobic bonding, the better terminology is probably "hydrophobic interaction" [3].

   Example: staining in aqueous solution and metachromatic staining.
6. *Dye aggregations*: Dye molecules may interact with each other forming dye-dye

interaction. They aggregate in solution and then penetrate into tissue. The dye-dye aggregate increases when the dye concentration is high, the molecular size of the dye is bigger and temperature is low.

7.3 Factors Influencing Staining

Several factors have influence on staining intensity (Box 7.2):

1. Dye affinity to the target tissue specimen
2. Specimen geometry
3. Target concentration
4. Rate of reaction
5. Rate of stain loss

1. *Dye affinity to the target tissue specimen*: The tendency to bind a dye with the target tissue is known as dye affinity. The acidic dye such as eosin binds strongly with acidophilic target, that is, cytoplasmic protein. The acidic dye has very little affinity with basophilic substances. Affinity of the dye to the specific tissue is also influenced by the pH and the presence of inorganic salt concentration of the solvent.
2. *Specimen geometry*: Specimen geometry or topography also influences the staining.
   (a) Thick tissue: If the tissue is thick, then the penetration of dye is difficult, and the central part of the tissue takes poorer stain.
   (b) Surface topography: The surface of the tissue of paraffin section is more even than cryostat section, and so it takes better stain.
   (c) Disturbance of microtopography of tissue: The alcohol is a coagulative fixative that disturbs the topography of the cell and tissue. The shattering effect of alcohol increases the dye penetration rate.
   (d) Inner geometry of tissue: The inner geometry of the tissue may also influence staining such as bone marrow canaliculi which are rapidly stained by Schmorl's thionine stain than the adjacent connective tissue.
3. *Target concentration*: The concentration of the target tissue affects the staining intensity as the more the amount of the target tissue, the more intense will be the staining.
4. *Rate of reaction*: Rate of reaction in the target tissue also influences the staining pattern such as in Feulgen reaction, the short reaction time exposes only a few aldehyde groups producing weak staining pattern.
5. *Rate of stain loss*: Staining pattern is greatly influenced by rate of stain loss. At times the stain loss may be intentional such as differentiation or destaining. The differentiation often removes the excess stain from the cell and thus helps to differentiate the organisms.

Box 7.2 Factors Influencing Staining Intensity

- *Dye affinity to the target tissue specimen*: Acid dye binds with positively charged acidophilic tissue, and basic dye binds with negatively charged basophilic tissue.
- *Specimen geometry*:
  - Thick tissue: Less penetration of dye.
  - Surface topography: More even surface gives better stain.
  - Microtopography of tissue: Alcohol fixation disturbs microtopography.
  - Inner geometry of tissue.
- *Target concentration*: The more the amount of the target tissue, the more intense will be the staining.
- *Rate of reaction*: Short reaction time often decreases stain intensity.
- *Rate of stain loss*: Too much differentiation often removes the stain.

7.3.1 Nomenclature Used Regarding Dye

Colour Index of Dye A certain dye may be called in different names, or the same name may be given to many different dyes. Therefore the simple name of a dye may evoke confusion. To avoid this confusion, the “society of dyers and colourist” has labelled a specific dye by the unique code known as colour index or colour index (CI) number. The CI number is actually a compendium of dye. With the help of the CI, one can identify the exact dye such as CI 52000 which indicates thionine.

7.4 Metachromasia [4]

The word “meta” means altered, and “chromasia” means colour, so “metachromasia” indicates altered colour. Metachromasia is defined as staining phenomenon when the tissue is stained in different colours from the original dye colour. The metachromatic dye is defined as the alteration of the original colour of the dye without any change of the chemical structure of the dye (Box 7.3).

7.4.1 Metachromatic Dyes

Cationic Dye Majority of the metachromatic dyes are positively charged cationic or basic dyes such as toluidine blue, methylene blue, azure A and B, methyl violet, brilliant cresyl blue, etc.

Anionic Dyes Only a few anionic dyes are metachromatic such as Biebrich scarlet and bromophenol blue. These anionic dyes are weakly metachromatic.

Mechanism of Metachromasia (Fig. 7.5) Glycosaminoglycans of the connective tissue and epithelial mucins and granules of mast cells are negatively charged polyanions. The cationic (positively charged) dye in aqueous solution reacts with the polyanions of the tissues. The binding of the dye molecule with these polyanions of the tissue neutralizes the positively charged dye. The nonpolar aromatic ring of the dye binds with the other dye by van der Waals force. The dye-dye aggregation occurs, and dimer, tetramer and polymer of the dye molecules are formed. Overall dye binding becomes stronger due to van der Waals force. The dye absorbs light of shorter wavelength, and the visible colour of the light emitted from the dye tissue changes. This causes metachromasia or altered colour, such as pyronin Y in the tissue that gives red to orange colour.

Box 7.3 Metachromasia

Metachromasia is a staining phenomenon when the tissue is stained in different colours from the original dye colour.

Metachromatic dyes

Cationic dye: Toluidine blue, methylene blue, azure A and B, methyl violet, brilliant cresyl blue, etc.

Anionic dyes: Biebrich scarlet and bromophenol blue.

Mechanism of metachromasia: The cationic (positively charged) dye in aqueous solution is neutralized with the polyanions of the tissues. Subsequently the nonpolar aromatic ring of the dye binds with the other dye by van der Waals force. The dye absorbs shorter wavelength of light, and the visible colour changes.

Various types of metachromasia:

- Alpha (orthochromatic): Blue colour
- Beta (di- and trimeric form): Purple colour
- Gamma (polymeric form): Red colour

Factors enhancing metachromasia:

- Higher dye concentration
- Low pH
- Decreased temperature
- Aqueous solution

Various Types of Metachromasia (Fig. 7.6) In relation to thiazine dye, three types of metachromatic change may be seen [5]:

- *Alpha (orthochromatic)*: The dye remains in monomeric form and gives blue colour.
- *Beta (di- and trimeric form)*: The dye forms dimeric structure and produces purple colour.

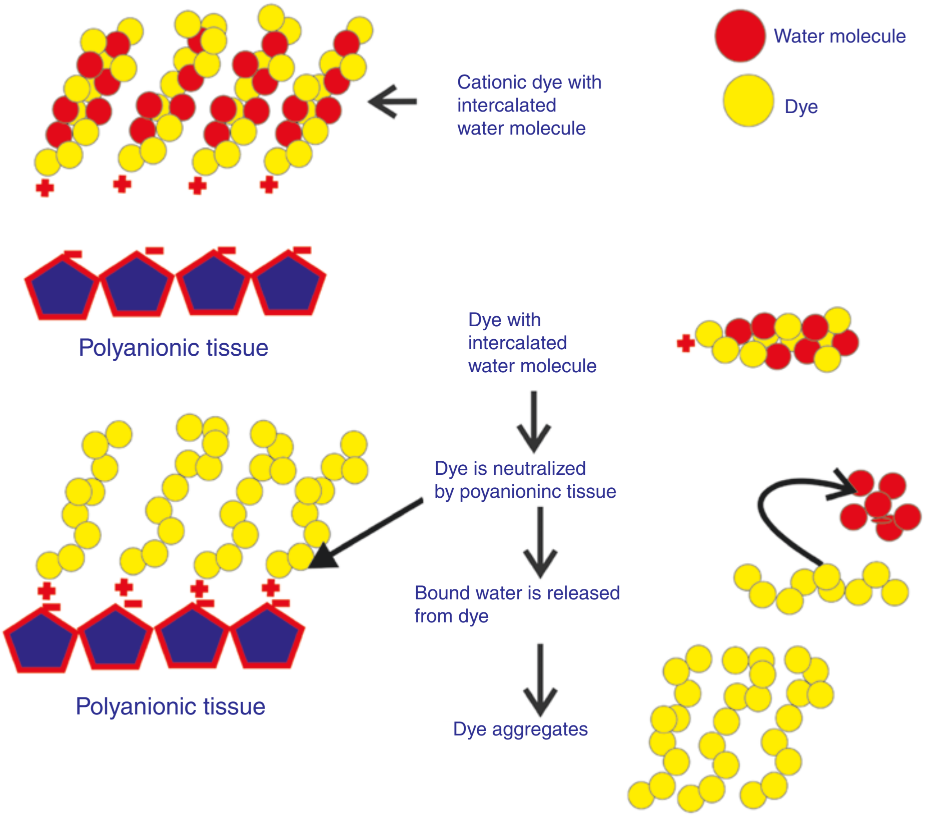
Water molecule
Dye
Cationic dye with intercalated water molecule
Polyanionic tissue
Dye with intercalated water molecule
Dye is neutralized by poyanioninc tissue
Bound water is released from dye
Dye aggregates
Polyanionic tissue

Fig. 7.5 Mechanism of dye aggregation in metachromasia. The cationic dye interacts with the polyanionic tissue, and the bound water of the dye molecule is released. The dye aggregates and the absorption of light changes by the aggregated dye-tissue complex

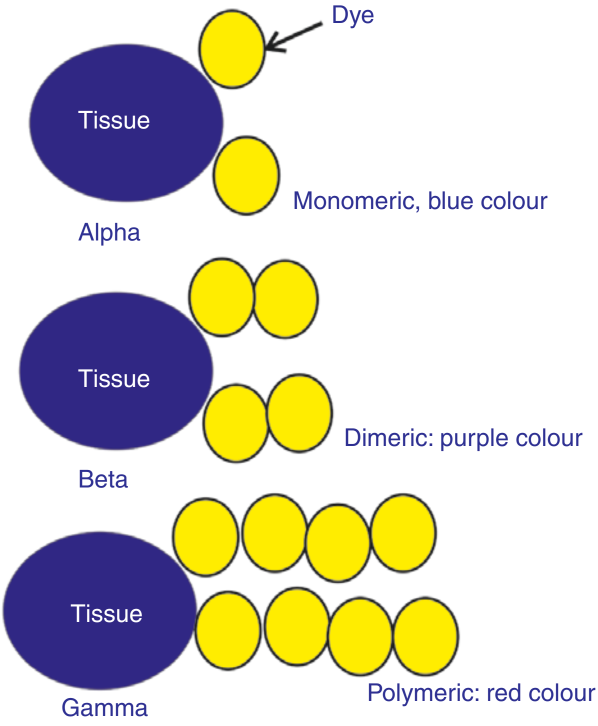

Fig. 7.6 The different types of metachromasia are highlighted in this schematic diagram

- *Gamma (polymeric form)*: The dye is in polymeric form and produces red colour.

The colour changes in metachromasia may not be homogenous. At times the anions in the tissue may be widely distributed. In such cases, there may be purple colouration of tissue due to the mixture of orthochromatic blue and polychromatic red colour.

Factors Influencing Metachromasia The following factors may influence on metachromasia:

1. Dye concentration: High concentration of dye enhances metachromasia.
2. pH: Low pH increases metachromatic effect.
3. Temperature: Decreased temperature augments metachromatic effect.
4. Aqueous solution: Water enhances van der Waals force in between the dye molecules and increases metachromatic effect.

7.5 Progressive and Regressive Staining

Progressive Staining In case of progressive staining, the dye is allowed to react with the tissue until it stains the target structure. In fact, this is a difficult task to supervise, and all other influencing factors should be controlled such as pH of the dye solution, thickness of tissue, concentration of dye, etc. It is always necessary to check the staining at frequent intervals to prevent overstaining or to have light staining.

Regressive Staining Here the tissue is intentionally overstained by dye. The affinity of the different structures of the tissue with dye is variable, and this particular property is exploited to remove the dye from the unwanted part of the tissue. This procedure is also known as *differentiation*. The differentiation is done by using:

(a) Acid in basic dye or base in acid dye such as in Papanicolaou's staining. Haematoxylin is removed from the cytoplasm by using 1% acid alcohol.
(b) Oxidizing agent: The oxidizing agents are used to oxidize the dye and make it a colourless material such as picric acid, potassium permanganate, etc.
(c) Mordant: Here the dye-mordant complex at first binds with the tissue. Subsequently excess mordant is used that attracts the attached dye in the tissue. The mordant thereby removes the excess dye from the tissue.
(d) One dye is replaced by other less affinity dye.

7.6 Mordant

Mordant is the salt and hydroxides of the metals that help in the attachment of dye with the target tissue (Box 7.4). The metal used as mordant is either divalent or trivalent such as aluminium, iron, copper, etc. The mordant binds with the dye by covalent or co-ordinate bonding. This dye and mordant combination is also known as "lake". The mordant-dye combination is basic in action irrespective of the character of the dye. Mordant is insoluble in most of the biological fluid, and

Box 7.4 Mordant

- It is the salt and hydroxides of the metals that help in the attachment of dye with the target tissue.
- It makes the dye strong.
- The mordant binds with the dye by covalent or co-ordinate bonding known as "lake".
- Type
  - Pre-mordanting: The tissue is at first treated with mordant followed by dye.
  - Meta-mordanting: Mordant in combination with dye is used.
  - Post-mordanting: The dye material is applied first followed by mordant.

  Example: aluminium and haematoxylin

therefore the staining is not altered even after subsequent treatment of the tissue.

Mordant may be used in three ways:

1. Pre-mordanting: The tissue is at first treated with mordant followed by dye.
2. Meta-mordanting: Mordant in combination with dye is used.
3. Post-mordanting: The dye material is applied first followed by mordant.

Example: Haematoxylin itself is a poor dye. However the combination of mordant such as aluminium and haematoxylin makes a stronger dye.

7.6.1 Accentuators

Accentuators are the group of substances that help to increase the staining intensity of the dye. Accentuators neither form any dye lake nor do they take part in any chemical reaction. The common example of accentuator is using potassium hydroxide in methylene blue solution.

7.7 Staining Procedure

The proper organization of the staining room is mandatory to get a well stained section. The staining room should be well ventilated and well illuminated (Box 7.5).

The workflow of histology/cytology laboratory is fixation, grossing, processing, mounting, microtomy and staining. Therefore the staining room should also be designed accordingly. The staining bench should be facing the window. The bench should be cleaned properly with arrangement of fume remover. There should be at least two supplies of running tap water with sink.

Box 7.5 Staining Room
- Arrange the room according to workflow.
- Do not congest the staining room.
- Clean room and work bench.
- Well ventilated and well illuminated.
- Running water with sink.

Stains and Equipment The reagents should be kept in the rack with proper arrangement and label (Box 7.6). The list of the reagents should be in the laboratory catalogue. The glass bottle is the best container to store the reagents. The use of amber-coloured bottle is preferable for the dye that reacts with light. Frequently used reagents can be kept in small glass bottle or Coplin jar. A microscope is necessary to check the stain. Automated strainer can stain large batches of slides containing more than 100 slides. Many laboratory prefers manual staining for small batches of slides. In that case, the glass troughs are used. It is preferable to use the series of sequential arrangement of glass troughs for staining. All the troughs should be well covered to prevent evaporation of the reagents particularly alcoholic solution. In addition the laboratory should have ample supply of distilled water.

The Preparation of the Staining Reagent The preparation of staining reagents is one of the most important tasks in any laboratory. Adequate cautions should be taken regarding cleaning glassware, using distilled water instead of tap water, following proper stepwise protocols to make the staining solution and maintaining the concentration of alcohol in alcoholic solution (Box 7.7).

Box 7.6 Reagents and equipment
- All reagents in the rack is labelled glass container.
- Maintain the readily available catalogue.
- Amber-coloured bottle for the reagents that needs protection from light.
- The glass troughs well covered.
- Ample supply of distilled water.
- Light microscope.

Box 7.7 Essential precautions during preparation of staining solution

- Clean glassware
- Distilled water instead of tap water
- Alcoholic solution to keep in airtight container
- Stepwise proper protocol to follow
- Fresh solution in case of ammonia solution
- Proper filtration of the staining solution before bacteriological stain
- Silver-containing solution to be kept in dark

7.7.1 Preparation of Buffer Solutions

7.7.1.1 Molar Solution

1M = one molar solution means molecular weight of the compound expressed in gramme dissolved in 1000 ml of water.

0.1M means one-tenth of the molecular weight of the compound in gramme dissolved in 1000 ml water.

7.7.1.2 Citrate Buffer

Citric acid: Dissolve 2.101 g of citric acid in 100 ml of distilled water.

Sodium citrate solution: Dissolve 2.9412 g sodium citrate in 100 ml of distilled water.

Final preparation: Now mix 46.5 ml of citric acid solution with 3.5 ml of sodium citrate solution. The distilled water is mixed in it to make up to 100 ml. This will make 0.1 M citrate buffer. The pH of the solution is adjusted to 2.5 with the help of pH metre by adding 1 N HCl and 5 N NaOH.

7.7.1.3 Phosphate Buffer

Stock A: 0.2M of sodium dihydrogen orthophosphate—Mix 31.2 g of sodium dihydrogen orthophosphate (molecular weight 156) in 1000 ml water.

Stock B: 0.2 m disodium hydrogen orthophosphate—Mix 28.3 g disodium hydrogen orthophosphate (molecular weight 142) in 1000 ml water.

To get pH 6: Mix 438 ml of stock A with 62 ml of stock B, and make up to 1000 ml by distilled water.

7.7.1.4 Tris-HCl Buffer

Stock A: 0.2M Tris.

Mix 2.42 g Tris(hydroxymethyl aminomethane) (of molecular weight 121) in 100 ml water.

Stock B: 0.2M HCl.

Mix 1.7 ml HCl (molecular weight 36.46) in 100 ml distilled water.

To get pH 7.2: Mix 25 ml of stock A with 22.1 ml of stock B, and make up to 100 ml by distilled water.

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